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Chimeric Cytoplasmic Signalling Molecules

The present invention relates to novel cytoplasmic signalling molecules, the nucleic acids encoding them and the use of these nucleic acids and cytoplasmic signalling molecules in medicine and research.

Throughout this application various publications are referenced by author and year of publication. Full citations for these publications are provided following the detailed description of the invention and examples.

CD28 is a co-stimulatory molecule constitutively expressed on the surface of CD4+ and 50% of CD8+ T cells and the interaction of CD28 with its ligands CD80 (B7.1) or CD86 (B7.2) augments T cell proliferation, IL-2 production and survival of naïve T cells. A number of additional costimulatory pathways have been recently reported and these fall into two superfamilies, CD28/B7 and TNF/TNFR. These costimulatory pathways appear to act at different stages of T cell differentiation and activation and they have been shown to play a role in promoting different effector functions.

The human inducible co-stimulator (ICOS) molecule is a member of the CD28/B7 superfamily and is a 55-60kDa disulphide-linked, glycosylated homodimer expressed on activated, but not resting, T cells (reviewed by Carreno & Collins, 2002). Ligation of ICOS enhances T cell proliferation and cytokine secretion; however, unlike CD28, it does not increase IL-2 production. ICOS cross-linking does promote a dramatic increase in the production of IL-10, a growth factor for B lymphocytes. ICOS binds a different ligand to CD28, ICOS-L (B7h, GL50, BRP-1, B7-H2, LICOS) although with a similar affinity to that of CD28 for CD80.

CD134, also known as OX40 is a member of the TNFR superfamily and is known to participate as a co-stimulatory molecule in T-cell activation. CD134 is a 48kDa glycoprotein expressed on activated (mainly CD4+) T cells one to two days after activation (Kjaergaard *et al.*, 2000). Expression is dependent upon signalling through the T cell receptor (TCR) complex and is enhanced by CD28 signalling (Rogers *et al.*, 2001). Co-stimulation via CD134 has been shown to enhance

proliferation and cytokine production, enhance tumour immunity and enhance memory T cell development. CD134 is thought to prolong the T cell response and promote long term survival and memory. CD134, like other members of the TNF receptor family associates with TNFR-associated factor (TRAF) signalling molecules, TRAF-2, -3 and –5 and activates NFkB.

Research in the area of immune cell signalling has yielded a considerable amount of information about the signal transduction events that occur downstream of antigen receptor engagement. A substantial number of studies have concentrated on the receptors themselves, and the enzymes stimulated in response to antigen binding (reviewed by Weiss & Littman, 1994; DeFranco, 1997).

Individual components of the T cell receptor (TCR) complex have been well characterised and in a number of cases the functionality of receptor sub-units or domains has been determined through the construction of chimeric receptor proteins (Kuwana *et al.,* 1987; Romeo *et al.*, 1992). Cytoplasmic signalling domains in particular, and their role in TCR activation, have been identified using this approach. This information has led to the development of chimeric receptors that are capable of regulating cell activation processes (see for example Finney *et al.*, 1998 and published International Patent Specifications WO 97/23613, WO 96/23814, WO 96/24671, WO 99/00494, WO 99/57268).

The ability to control the biological effects of cellular activation, for example, increased cellular proliferation, increased expression of cytokines, stimulation of cytolytic activity, differentiation of other effector functions, antibody secretion, phagocytosis, tumor infiltration and/or increased cellular adhesion, with chimeric receptors has considerable therapeutic potential.

Whilst currently available chimeric receptors are capable of effectively activating cells, there is room for improvement in the efficacy with which the cytoplasmic signalling domain of such a chimeric receptor transduces the signal from the extracellular ligand binding domain to downstream members of secondary messenger pathways. There is also a need to provide alternative cytoplasmic signalling domains which enhance and prolong the T cell response over that

provided by CD28. Alternative cytoplasmic signalling domains are also required to achieve effective signalling in situations where CD28 signalling is insufficient or absent. For example, $CD8^+CD28^-$ T cells which are prevalent in a number of situations ranging from chronic inflammatory conditions and infectious diseases to ageing and immunodeficiency (Arosa, 2002) no longer express CD28 on their surface and may no longer be able to signal through the CD28 pathway. In certain situations it may be desirable to provide co-stimulatory signalling in these cells and this may require co-stimulation through alternative pathways.

Successful expression and signalling of a fusion receptor with both TCRζ and CD28 signalling regions in a single molecule have been demonstrated in Jurkat (Finney *et al.,* 1998) and in cultured primary human T cells (Eshar *et al.,* 2001; Hombach *et al.*, 2001; Maher *et al.*, 2002). However, transfection of chimeric receptors into human primary T cells has until recently been limited to activated primary T cells as retroviral transduction requires cells to be in cell cycle (Miller *et al.*, 1990). As a result, no expression or function of co-stimulation receptors has been reported in resting human primary T cells. It is not yet known whether receptors containing signalling regions that would not normally be expressed in resting T cells are able to mediate signalling through their normal pathways.

The current invention addresses these difficulties by providing novel chimeric receptors and novel cytoplasmic signalling molecules that comprise at least part of the CD134 or ICOS polypeptides and are capable of activating resting T cells. The current invention also provides novel cytoplasmic signalling molecules capable of enhancing and prolonging T cell responses and mediating cellular activation more efficiently by transducing signals through more than one secondary messenger pathway.

Surprisingly, we have been able to demonstrate that the use of the CD134 or ICOS polypeptides as part of a chimeric receptor, results in cellular activation in response to extracellular ligand binding to the receptor when expressed in resting T cells in the absence of CD28 signalling. We have been able to demonstrate that co-stimulation of resting T cells occurs both when the primary activation signal is provided by stimulation of the endogenous TCR complex and when the primary

activation signal sequence is part of the same chimeric receptor. In particular, we have demonstrated that where CD134 or ICOS is employed in conjunction with at least one other cytoplasmic signalling sequence to form a cytoplasmic signalling molecule, this can confer novel and unexpected properties on the cytoplasmic signalling molecule. For example, where a cytoplasmic signalling molecule comprising CD134 or ICOS in conjunction with at least one further cytoplasmic signalling sequence is employed as the cytoplasmic signalling domain of a chimeric receptor and expressed in resting T cells, the resulting levels of cellular activation are much higher than would be predicted.

Thus according to one aspect of the present invention there is provided a nucleic acid encoding a chimeric receptor protein, which comprises an extracellular ligand-binding domain, a transmembrane domain and a cytoplasmic signalling domain, comprising a cytoplasmic signalling molecule derived from CD134 or ICOS.

In one embodiment, the invention provides a nucleic acid encoding a chimeric receptor protein, which comprises an extracellular ligand-binding domain, a transmembrane domain and a single cytoplasmic signalling domain, wherein the cytoplasmic signalling domain comprises a single cytoplasmic signalling sequence derived from CD134 or ICOS.

In another aspect of the present invention there is provided a nucleic acid encoding a cytoplasmic signalling molecule comprising at least two cytoplasmic signalling sequences, wherein at least one cytoplasmic signalling sequence is derived from CD134 or ICOS. Preferably, a cytoplasmic signalling sequence of the invention will transduce signals via the tumor necrosis factor receptor pathways or via the B7/CD28 signalling pathways. In one aspect of the present invention each cytoplasmic signalling sequence mediates signal transduction through a different secondary messenger pathway.

In one embodiment, there is provided a nucleic acid encoding a cytoplasmic signalling molecule comprising at least two cytoplasmic signalling sequences wherein one cytoplasmic signalling sequence is derived from CD134 or ICOS.

The term 'cytoplasmic signalling sequence' as used herein, means any sequence of amino acids that form at least a substantial part of a larger domain and are known to function as a unit capable of transducing a signal, which results in the activation or inhibition of biological processes within a cell.

The CD134 and ICOS polypeptides consist of an extracellular domain, a transmembrane domain and a cytoplasmic domain that is responsible for intracellular signal transduction. Accordingly a cytoplasmic signalling sequence derived from CD134 or ICOS will comprise the cytoplasmic domain or a derivative or variant thereof that has the same functional capability. The terms 'derivative' or 'variant' mean any species variant or any variant comprising one or more amino acid substitution, deletion or addition, provided that the derivative or variant retains substantially the same functional capability as the original parent sequence. Preferably cytoplasmic signalling molecules of the invention will contain a cytoplasmic signalling sequence comprising amino acid residues 166 to 199 of ICOS (Hutloff *et al.*, 1999), or residues 213 to 249 of CD134 (Latza *et al.*, 1994) or a derivative or variant thereof.

Preferred examples of further cytoplasmic signalling sequences for use in the invention include the cytoplasmic sequences of the TCR and co-receptors that act in concert to initiate signal transduction following antigen receptor engagement, as well as any derivative or variant (as described above) of these sequences and any synthetic sequence that has the same functional capability.

It is known that signals generated through the TCR alone are insufficient for full activation of the T cell and that a secondary or co-stimulatory signal is also required. Thus, T cell activation can be said to be mediated by two distinct classes of cytoplasmic signalling sequence: those that initiate antigen-dependent primary activation through the TCR (primary cytoplasmic signalling sequences) and those that act in an antigen-independent manner to provide a secondary or co-stimulatory signal (secondary cytoplasmic signalling sequences).

In one aspect of the present invention a cytoplasmic signalling molecule will comprise at least one cytoplasmic signalling sequence derived from CD134 or ICOS and at least one primary cytoplasmic signalling sequence.

Primary cytoplasmic signalling sequences regulate primary activation of the TCR complex either in a stimulatory way, or in an inhibitory way. Primary cytoplasmic signalling sequences that act in a stimulatory manner may contain signalling motifs which are known as immunoreceptor tyrosine-based activation motifs or ITAMs (Reth, 1989), whereas those that act in an inhibitory manner may contain signalling motifs known as immunoreceptor tyrosine-base inhibition motifs or ITIMs (Burshtyn *et al.*, 1999).

Thus primary cytoplasmic signalling sequences for use in this or any aspect of the invention described herein, will preferably contain either an immunoreceptor tyrosine-based activation motif (ITAM), or an immunoreceptor tyrosine-based inhibitory motif (ITIM).

Examples of ITAM containing primary cytoplasmic signalling sequences that are of particular use in the invention include those derived from TCRζ, FcRγ, FcRβ, CD3γ, CD3δ, CD3ε, CD5, CD22, CD79a, CD79b, and CD66d. Preferably cytoplasmic signalling sequences derived from these molecules will comprise amino acid residues 31-142 of TCRζ, amino acids residues 27-68 of FcRγ, amino acid residues 201-244 of FcRβ, amino acid residues 117-160 of CD3γ, amino acid residues 107-150 of CD3δ, amino acid residues 131-185 of CD3ε, amino acid residues 378-471 of CD5, amino acid residues 688-828 of CD22, amino acid residues 134-194 of CD79a, amino acid residues 154-201 of CD79b, or residues 143-218 of CD66d.

It is particularly preferred that cytoplasmic signalling molecules according to this aspect of the invention comprise a cytoplasmic signalling sequence derived from TCRζ.

In an alternative embodiment, a cytoplasmic signalling molecule of the invention will comprise at least one cytoplasmic signalling sequence derived from CD134 or ICOS and at least one other secondary cytoplasmic signalling sequence.

Molecules containing secondary cytoplasmic signalling sequences suitable for use in this or any aspect of the invention described herein include CD2, CD4, CD5, CD8α, CD8β, CD28, CD137, CD134, ICOS, and CD154. Preferably cytoplasmic signalling sequences derived from these molecules will comprise amino acid residues 212-327 of CD2, amino acid residues 396-433 of CD4, amino acid residues 378-471 of CD5, amino acid residues 186-214 of CD8α, amino acid residues 175-189 of CD8β, amino acid residues 162-202 of CD28, amino acid residues 214-255 of CD137, amino acid residues 213-249 of CD134, amino acid residues 166-199 of ICOS or amino acid residues 1-22 of CD154. It is particularly preferred that secondary signalling sequences derived from CD28 are employed in conjunction with a cytoplasmic signalling sequence derived from CD134 or ICOS.

We have also found that a cytoplasmic signalling molecule comprising CD134 or ICOS in conjunction with two further cytoplasmic signalling molecules is particularly efficient at mediating signal transduction when employed as the cytoplasmic signalling domain of a chimeric receptor.

A further aspect of the invention therefore provides a nucleic acid encoding a cytoplasmic signalling molecule comprising at least one cytoplasmic signalling sequence derived from CD134 or ICOS and at least two further cytoplasmic signalling sequences.

One or both of the at least two additional cytoplasmic signalling sequences may be either a primary cytoplasmic signalling sequence or a secondary cytoplasmic signalling sequence as described hereinbefore. However, it is especially preferred that at least one additional cytoplasmic signalling sequence will be a primary cytoplasmic sequence and at least one other cytoplasmic signalling sequence will be a secondary cytoplasmic signalling sequence. Whilst any of the primary and secondary cytoplasmic signalling sequences described above may be incorporated

into a cytoplasmic signalling molecule according to this aspect of the invention, the combination of a primary signalling sequence derived from TCRζ with a secondary signalling sequence derived from CD28 is preferred.

The cytoplasmic signalling sequences within a cytoplasmic signalling molecule of the invention may be linked to each other in a random or specified order. Optionally, a short oligo- or polypeptide linker, preferably between 2 and 10 amino acids in length may form the linkage. A glycine-serine doublet provides a particularly suitable linker.

Nucleic acids encoding cytoplasmic signalling molecules comprising cytoplasmic CD134 or ICOS and one additional cytoplasmic signalling sequence may thus encode in reading frame: i) a cytoplasmic signalling sequence derived from CD134 or ICOS and ii) a primary cytoplasmic signalling sequence; i) a primary cytoplasmic signalling sequence and ii) a cytoplasmic signalling sequence derived from CD134 or ICOS; i) a cytoplasmic signalling sequence derived from CD134 or ICOS and ii) a secondary cytoplasmic signalling sequence; or i) a secondary cytoplasmic signalling sequence and ii) a cytoplasmic signalling sequence derived from CD134 or ICOS. Specific examples of such cytoplasmic signalling molecules include those that comprise, in order from the amino to carboxyl terminus, cytoplasmic signalling sequences derived from i) CD134 or ICOS and ii) TCRζ; i) TCRζ and ii) CD134 or ICOS; i) CD134 or ICOS and ii) CD28; and i) CD28 and ii) CD134 or ICOS; i) CD134 and ii) ICOS; i) ICOS and ii) CD134.

Where cytoplasmic signalling molecules of the invention comprise a cytoplasmic signalling sequence derived from CD134 or ICOS and at least two additional cytoplasmic signalling sequences, these may also be linked in random or specified order, optionally via a linker as described above. Thus nucleic acids encoding such cytoplasmic signalling molecules may encode i) a cytoplasmic signalling sequence derived from CD134 or ICOS, ii) a primary cytoplasmic signalling sequence and iii) a secondary cytoplasmic signalling sequence; i) a cytoplasmic signalling sequence derived from CD134 or ICOS, ii) a secondary cytoplasmic signalling sequence and iii) a primary cytoplasmic signalling sequence; i) a primary cytoplasmic signalling sequence, ii) a cytoplasmic signalling sequence derived from CD134 or ICOS and

iii) a secondary cytoplasmic signalling sequence; i) a primary cytoplasmic signalling sequence, ii) a secondary cytoplasmic signalling sequence and iii) a cytoplasmic signalling sequence derived from CD134 or ICOS; i) a secondary cytoplasmic signalling sequence ii) a primary cytoplasmic signalling sequence and iii) a cytoplasmic signalling sequence derived from CD134 or ICOS; or i) a secondary cytoplasmic signalling sequence, ii) a cytoplasmic signalling sequence derived from CD134 or ICOS and iii) a primary cytoplasmic signalling sequence.

Specific examples of such cytoplasmic signalling molecules include those that comprise, in order from the amino to carboxyl terminus, cytoplasmic signalling sequences derived from i) CD134 or ICOS, ii) TCRζ and iii) CD28; i) CD134 or ICOS, ii) CD28 and iii) TCRζ; i) TCRζ, ii) CD134 or ICOS and iii) CD28; i) TCRζ, ii) CD28 and iii) CD134 or ICOS; i) CD28, ii) TCRζ and iii) CD134 or ICOS; and i) CD28, ii) CD134 or ICOS and iii) TCRζ. Specific examples also include cytoplasmic signalling molecules that comprise both CD134 and ICOS combined with TCRζ or CD28 in any order.

The novel cytoplasmic signalling molecules of the invention can be used, either by themselves or, as a component part of a larger protein such as a chimeric receptor. As individual protein molecules, they can be introduced into, or expressed in, effector cells in order to act as substitute cytoplasmic signalling sequences for immune cell receptors already expressed within that cell. In this way they can increase the efficiency of signalling through the receptor and prolong or enhance the T cell response. They may exist as soluble polypeptides in the cell cytoplasm, or they may be anchored or tethered to a cell membrane and extend into the cytoplasm, where they are capable of mediating signal transduction under a given set of physiological cellular conditions.

However, it is envisaged that the cytoplasmic signalling molecules of this invention are used preferentially to mediate signalling when employed as a cytoplasmic signalling domain of a chimeric receptor protein. Such chimeric receptors also comprise an extracellular ligand-binding domain and a transmembrane domain.

Thus, according to this aspect of the invention there is provided a nucleic acid encoding a chimeric receptor protein comprising an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic signalling domain comprising a single cytoplasmic signalling sequence derived from CD134 or ICOS. In one embodiment the cytoplasmic signalling domain mediates signal transduction through at least two different secondary messenger pathways.

The incorporation of an extracellular ligand-binding domain confers on the chimeric receptor the ability to exhibit specificity for a specific ligand or class of ligands. This specificity can be used to define precise ligands or classes of ligands that are capable of activating the receptor. In this way the receptor may be designed to activate the cell in which it is expressed upon binding a chosen class of, or individual, ligand.

Contact between the ligand and its corresponding binding domain in a chimeric receptor, results in signal transduction through the cytoplasmic signalling domain. The combination of cytoplasmic signalling sequences within the cytoplasmic signalling molecule of the invention chosen to act as a cytoplasmic domain of the chimeric receptor, dictates the magnitude of the signal transduced, and consequently controls the level to which the cell is activated.

A further embodiment of the invention thus provides nucleic acids encoding chimeric receptor proteins comprising an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic signalling domain wherein the cytoplasmic signalling domain is encoded by a nucleic acid encoding a cytoplasmic signalling molecule according to any of the previously described aspects of the invention.

The term "extracellular ligand-binding domain" as used herein, is defined as any oligo- or polypeptide that is capable of binding a ligand. Accordingly antibody binding domains, antibody hypervariable loops or CDRs, receptor binding domains and other ligand binding domains, examples of which will be readily apparent to the skilled artisan, are described by this term. Preferably the domain will be capable of interacting with a cell surface molecule. Examples of proteins associated with

binding to cell surface molecules that are of particular use in this invention include, antibody variable domains (V_H or V_L), T-cell receptor variable region domains (TCRα, TCRβ, TCRγ, TCRδ), or the chains of CD8α, CD8b, CD11A CD11B, CD11C, CD18, CD29, CD49A, CD49B, CD49D, CD49E, CD49F, CD61, CD41, or CD51. Whilst it may be of benefit to use the entire domain or chain in some instances, fragments may be used where appropriate. Fab' fragments or, especially single chain Fv fragments, are particularly useful binding components.

The choice of domain will depend upon the type and number of ligands that define the surface of a target cell. For example, the extracellular ligand binding domain may be chosen to recognise a ligand that acts as a cell surface marker on target cells associated with a particular disease state. Thus examples of cell surface markers that may act as ligands include those associated with viral, bacterial and parasitic infections, autoimmune disease and cancer cells. In the latter case, specific examples of cell surface markers are the bombesin receptor expressed on lung tumour cells, carcinoembryonic antigen (CEA), polymorphic epithelial mucin (PEM), CD33, the folate receptor, epithelial cell adhesion molecule (EPCAM) and *erb*-B2. Other ligands of choice are cell surface adhesion molecules, inflammatory cells present in autoimmune disease, and T cell receptors or antigens that give rise to autoimmunity. The potential ligands listed above are included by way of example; the list is not intended to be exclusive and further examples will be readily apparent to those of skill in the art.

Chimeric receptors may be designed to be bi- or multi-specific i.e. they may comprise more than one ligand binding domain and therefore, be capable of exhibiting specificity for more than one ligand. Such receptors may recruit cellular immune effector cells (e.g. T cells, B cells, natural killer (NK) cells, macrophages, neutrophils, eosinophils, basophils, or mast cells), or components of the complement cascade.

A further component of a chimeric receptor is the transmembrane domain. This may be derived either from a natural or from a synthetic source. Where the source is natural, the domain may be derived from any membrane–bound or transmembrane protein. Transmembrane regions of particular use in this invention

may be derived from (i.e. comprise at least the transmembrane region(s) of) the α, β or ζ chain of the T-cell receptor, CD28, CD3ε, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, ICOS, CD154. Alternatively the transmembrane domain may be synthetic, in which case it will comprise predominantly hydrophobic residues such as leucine and valine. Preferably a triplet of phenylalanine, tryptophan and valine will be found at each end of a synthetic transmembrane domain. Optionally, a short oligo- or polypeptide linker, preferably between 2 and 10 amino acids in length may form the linkage between the transmembrane domain and the cytoplasmic signalling domain of the chimeric receptor. A glycine-serine doublet provides a particularly suitable linker.

Between the extracellular ligand-binding domain and the transmembrane domain, or between the cytoplasmic signalling domain and the transmembrane domain, there may be incorporated a spacer domain. As used herein, the term "spacer domain" generally means any oligo- or polypeptide that functions to link the transmembrane domain to, the extracellular ligand-binding domain and/or, the cytoplasmic signalling domain in the polypeptide chain. A spacer domain may comprise up to 300 amino acids, preferably 10 to 100 amino acids and most preferably 25 to 50 amino acids.

Spacer domains may be derived from all or part of naturally occurring molecules, such as from all or part of the extracellular region of CD8, CD4, or CD28, or from all or part of an antibody constant region. Alternatively, the spacer may be a synthetic sequence that corresponds to a naturally occurring spacer sequence, or may be an entirely synthetic spacer sequence.

Spacer domains may be designed in such a way that they, either minimise the constitutive association of chimeric receptors, thus reducing the incidence of constitutive activation in the cell or, promote such associations and enhance the level of constitutive activation in the cell. Either possibility may be achieved artificially by deleting, inserting, altering or otherwise modifying amino acids and naturally occurring sequences in the transmembrane and/or spacer domains, which have side chain residues that are capable of covalently or non-covalently interacting with the side chains of amino acids in other polypeptide chains. Particular examples of amino acids that can normally be predicted to promote association include

cysteine residues, charged amino acids or amino acids such as serine or threonine within potential glycosylation sites.

Chimeric receptors may be designed in such a way that the spacer and transmembrane components have free thiol groups, thereby providing the receptor with multimerisation, and particularly dimerisation, capacity. Such multimeric receptors are preferred, especially dimers. Receptors with spacer domains derived from CD28 components and/or antibody hinge sequences and transmembrane regions derived from CD28 and the zeta chain of the natural T cell receptor are especially preferred.

The current invention not only provides the nucleic acids encoding novel cytoplasmic signalling molecules and chimeric receptor proteins, but also extends to the proteins themselves.

Nucleic acid coding sequences of the cytoplasmic signalling sequences for use in this invention, are readily derived from the specified amino acid sequences. Other nucleic acid sequences are widely reported in the scientific literature and are also available in public databases. DNA may be commercially available, may be part of cDNA libraries, or may be generated using standard molecular biology and/or chemistry procedures as will be clear to those of skill in the art. Particularly suitable techniques include the polymerase chain reaction (PCR), oligonucleotide-directed mutagenesis, oligonucleotide-directed synthesis techniques, enzymatic cleavage or enzymatic filling-in of gapped oligonucleotide. Such techniques are described by Sambrook & Fritsch 1989, and in the examples contained hereinafter.

The nucleic acids of the invention may be used with a carrier. The carrier may be a vector or other carrier suitable for the introduction of the nucleic acids *ex-vivo* or *in-vivo* into target cell and/or target host cells. Examples of suitable vector include viral vectors such as retroviruses, adenoviruses, adeno-associated viruses (AAVs), Epstein-Barr virus (EBV) and Herpes simplex virus (HSV). Non-viral vector may also be used, such as liposomal vectors and vectors based on condensing agents such as the cationic lipids described in International patent application numbers WO96/10038, WO97/18185, WO97/25329, WO97/30170 and WO97/31934. Where

appropriate, the vector may additionally include promoter and regulatory sequences and/or replication functions from viruses, such as retrovirus long terminal repeats (LTRs), AAV repeats, SV40 and human cytomegalovirus (hCMV) promoters and/or enhancers, splicing and polyadenylation signals and EBV and BK virus replication functions. Tissue-specific regulatory sequences such as the TCR-α promoter, E-selectin promoter and the CD2 promoter and locus control region may also be used. The carrier may be an antibody.

The invention also includes cloning and expression vectors containing a nucleic acid according to any of the above-described aspects of the invention. Such expression vectors will incorporate the appropriate transcriptional and translation control sequences, for example, enhancer elements, promoter-operator regions, termination stop sequence, mRNA stability sequences, start and stop codons or ribosome binding sites, linked where appropriate in-frame with the nucleic acid molecules of the invention.

Additionally in the absence of a naturally effective signal peptide in the protein sequence, it may be convenient to cause recombinant cytoplasmic signalling proteins to be secreted from certain hosts. Accordingly, further components of such vectors may include nucleic acid sequences encoding secretion signalling and processing sequences.

Vectors according to the invention include plasmids and viruses (including both bacteriophage and eukaryotic viruses). Many expression systems suitable for the expression of heterologous proteins are well known and documented in the art. For example, the use of prokaryotic cells such as *Escherichia coli* to express heterologous polypeptides and polypeptide fragments is well established (see for example, Sambrook & Fritsch, 1989, Glover, 1995a). Similarly, eukaryotic expression systems have been well developed and are commonly used for heterologous protein expression (see for example, Glover, 1995b and O'Reilly *et al.*, 1993). In eukaryotic cells, apart from yeasts, the vectors of choice are virus-based. Particularly suitable viral vectors include baculovirus-, adenovirus-, and vaccinia virus-based vectors.

Vectors containing the relevant regulatory sequences (including promoter, termination, polyadenylation, and enhancer sequences, marker genes) can either be chosen from those documented in the literature, or readily constructed for the expression of the proteins of this invention using standard molecular biology techniques. Such techniques, and protocols for the manipulation of nucleic acids, for example in the preparation of nucleic acid constructs, mutagenesis, sequencing, DNA transformation and gene expression, as well as the analysis of proteins, are described in detail in Ausubel *et al.*, 1992 or Rees *et al.*, 1993.

Suitable host cells for the *in vitro* expression of the cytoplasmic signalling molecules and chimeric receptor proteins of the invention include prokaryotic cells e.g. *E. coli*, eukaryotic yeasts e.g *Saccharomyces cerevisiae*, *Pichia* species, *Schizosaccharomyces pombe*, mammalian cell lines and insect cells. Alternatively chimeric receptors of the invention may be expressed *in vivo* in a variety of host such as, for example, insect larvae, plant cells, or more preferably mammalian tissues.

Nucleic acid may be introduced into a host cell by any suitable technique. In eukaryotic cells these techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, particle bombardment, liposome-mediated transfection or transduction using retrovirus, adenovirus or other viruses, such as vaccinia or, for insect cells, baculovirus. In bacterial cells, suitable techniques may include calcium chloride transformation, electroporation or transfection using bacteriophage. The nucleic acid may remain in an episomal form within the cell, or it may integrate into the genome of the cell. If the latter is desired, sequences that promote recombination with the genome will be included in the nucleic acid. Following introduction of the nucleic acid into host cells, the cells may be cultured under conditions to enhance or induce expression of the chimeric receptor protein as appropriate.

Thus, further aspects of the invention provide host cells containing a nucleic acid encoding a cytoplasmic signalling molecule and/or chimeric receptor protein as described herein, and host cells expressing such proteins.

According to still further aspects, the nucleic acids of the invention can be employed in either *ex-vivo* or *in-vivo* therapies.

For *ex-vivo* use the nucleic acid may be introduced into effector cells (removed from the target host) using methods well known in the art e.g. transfection, transduction (including viral transduction), biolistics, protoplast fusion, calcium phosphate mediated DNA transformation, electroporation, cationic lipofection, or targeted liposomes. The effector cells are then reintroduced into the host using standard techniques. Examples of suitable effector cells for the expression of the adaptor receptors of the present invention include cells associated with the immune system such as lymphocytes e.g. cytotoxic T-lymphocytes, tumour infiltrating lymphocytes, neutrophils, basophils, or T-helper cells, dendritic cells, B-cells, haematopoietic stem cells, macrophages, monocytes or NK cells. The use of cytotoxic T-lymphocytes is preferred. The use of resting T-lymphocytes is especially preferred.

Nucleic acids of the invention are particularly suitable for *in vivo* administration. In order to achieve this, the nucleic acid, preferably DNA, may be in the form of a targeted carrier system in which a carrier as described above is capable of directing the nucleic acid to a desired effector cell. Examples of suitable targeted delivery systems include targeted naked DNA, targeted liposomes encapsulating and/ or complexed with the DNA, targeted retroviral systems and targeted condensed DNA such as protamine and polylysine-condensed DNA.

Targeting systems are well known in the art and include, for example, using antibodies or fragments thereof against cell surface antigens expressed on target cells in vivo such as CD8, CD16, CD4, CD3, selectins (e.g. E-selectin), CD5, CD7, CD24, and activation antigens (e.g. CD69 and IL-2R. Alternatively other receptor-ligand interactions can be used for targeting e.g. CD4 to target $HIV_{gp}160$-expressing target cells.

In general, the use of antibody-targeted DNA is preferred, particularly antibody-targeted naked DNA, antibody-targeted condensed DNA and especially antibody-targeted liposomes. Types of liposomes that may be used include for example pH-sensitive liposomes, where linkers that are cleaved at low pH may be used to link

the antibody to the liposome. The nucleic acids of the present invention may also be targeted directly to the cytoplasm by using cationic liposomes, which fuse with the cell membrane. Liposomes for use in the invention may also have hydrophilic molecules, e.g. polyethylene glycol polymers, attached to their surface to increase their circulating half-life. There are many example in the art of suitable groups for attaching DNA to liposomes or other carriers; see for example International patent application numbers WO88/04924, WO90/09782, WO91/05545, WO91/05546, WO93/19738, WO94/20073 and WO94/22429. The antibody or other targeting molecule may be linked to the DNA, condensed DNA or liposome using conventional linking groups and reactive functional groups in the antibody, e.g. thiols or amines, and in the DNA or DNA-containing material.

Non-targeted carrier systems may also be used. In these systems targeted expression of the protein is advantageous. This may be achieved, for example, by using T cell specific promoter systems such as the zeta promoter, CD2 promoter and locus control region, CD4, CD8 TCRα and TCRβ promoters, cytokine promoters, such as the IL2 promoter, and the perforin promoter.

It is intended that the cytoplasmic signalling molecules and chimeric receptor proteins of the present invention, or the nucleic acids encoding them, be applied in methods of therapy of mammalian, particularly human, patients. Cytoplasmic signalling molecules and chimeric receptors generated by the present invention may be particularly useful in the treatment of a number of diseases or disorders. Such diseases or disorders may include those described under the general headings of infectious diseases, e.g. HIV infection; inflammatory disease/autoimmunity e.g. asthma, eczema; congenital e.g. cystic fibrosis, sickle cell anaemia; dermatologic, e.g. psoriasis; neurologic, e.g. multiple sclerosis; transplants e.g. organ transplant rejection, graft-versus-host disease; metabolic/idiopathic disease, e.g. diabetes; cancer.

For example, expression of a chimeric receptor of the invention on the surface of a T cell may initiate the activation of that cell upon binding of the ligand-binding domain to a ligand on a target cell. The ensuing release of inflammatory mediators

stimulated by the activation of the signalling function of the receptor ensures destruction of the target cell.

When a chimeric receptor according to the present invention is expressed in an effector cell of the immune system, binding to target will activate the effector cell; downstream effects of this activation may also result in the destruction of the target cell. If the extracellular ligand-binding domain of the chimeric receptor exhibits specificity for a surface marker on an immune cell, effector cells may be recruited to the site of disease. Accordingly, expression of a chimeric receptor of the invention in a diseased cell will ensure its destruction.

The expression of multispecific chimeric receptor proteins, or more than one chimeric receptor (with different ligand specificities), within a single host cell, may confer dual functionality on the receptor. For example, binding of the chimeric receptor to its target may not only activate the effector cell itself, but may additionally attract other immune effectors to the site of disease. The target cell may thus be destroyed by the activation of the immune system.

The cytoplasmic signalling molecules and chimeric receptor proteins of the present invention, or nucleic acids encoding them may also be used to provide co-stimulatory signalling to senescent T cells for example $CD8^+$ $CD28^-$ T cells which are prevalent in a number of situations such as inflammatory conditions and ageing (Arosa, 2002). Such cells no longer express CD28 on their surface and have a low proliferative capacity and may no longer have a functional CD28 signalling pathway. In some instances it may be desirable to provide these cells with a co-stimulatory signal and that may require a co-stimulatory signal that signals through a different pathway, such as ICOS or CD134.

A further aspect of the invention provides a composition comprising a cytoplasmic signalling molecule, or a chimeric receptor protein, or a nucleic acid(s) encoding a cytoplasmic signalling molecule or chimeric receptor protein, according to any of the aspects of the invention described above, in conjunction with a pharmaceutically acceptable excipient.

Suitable excipients will be well known to those of skill in the art and may, for example, comprise a phosphate-buffered saline (e.g. 0.01M phosphate salts, 0.138M NaCl, 0.0027M KCl, pH7.4), a liquid such as water, saline, glycerol or ethanol, optionally also containing mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulphates and the like; and the salts of organic acids such as acetates propionates, malonates, benzoates and the like. Auxiliary substances such as wetting or emulsifying agents, and pH buffering substances, may also be present. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co., N.J. 1991). Preferably, the compositions will be in a form suitable for parenteral administration e.g. by injection or infusion, for example by bolus injection or continuous infusion or particle-mediated injection. Where the composition is for injection or infusion, it may take the form of a suspension, solution or emulsion in an oily or aqueous vehicle and it may contain formulatory agents such as suspending, preservative, stabilising and/or dispersing agents. Alternatively, the composition may be in dry form, for reconstitution before use with an appropriate sterile liquid. For particle-mediated administration, DNA may be coated on particles such as microscopic gold particles.

A carrier may also be used that does not itself induce the production of antibodies harmful to the individual receiving the composition and which may be administered without undue toxicity. Suitable carriers are typically large, slowly metabolised macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers and inactive virus particles. Pharmaceutical compositions may also contain preservatives in order to prolong shelf life in storage.

If the composition is suitable for oral administration, the formulation may contain, in addition to the active ingredient additives such as starch (e.g. potato, maize or wheat starch, cellulose), starch derivatives such as microcrystalline cellulose, silica, various sugars such as lactose, magnesium carbonate and/or calcium phosphate. It is desirable that a formulation suitable for oral administration be well tolerated by the patient's digestive system. To this end, it may be desirable to include mucus formers and resins. It may also be desirable to improve tolerance by formulating the

compositions in a capsule that is insoluble in the gastric juices. In addition, it may be preferable to include the composition in a controlled release formulation.

According to yet a further aspect of the invention the use of the nucleic acids encoding novel cytoplasmic signalling molecules or chimeric receptor proteins, or of the polypeptides so encoded, or of a pharmaceutical composition containing such nucleic acids or polypeptides, in the manufacture of a medicament for the treatment or prevention of disease in humans or in animals is also provided.

The various aspects and embodiments of the present invention will now be illustrated in more detail by way of example. It will be appreciated that modification of detail may be made without departing from the scope of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Cloning cassette for construction of chimeric receptors comprising cytoplasmic signalling domains containing a CD134 or ICOS-derived cytoplasmic signalling sequence, and control chimeric receptors.

Figure 2: IL-2 production elicited by signalling via ICOS and CD134 co-stimulation receptors expressed in resting human $CD4^+$ T cells. Primary stimulation of endogenous CD3 is provided by plate bound OKT3 and co-stimulation is provided by the co-stimulation receptors via plate bound CD33.

Figure 3: Cytokine production elicited by antigen specific stimulation of chimeric receptor proteins expressed in resting $CD8^+$ human primary T cells having cytoplasmic signalling domains comprising a cytoplasmic signalling sequence derived from CD134 or ICOS in addition to a TCRζ cytoplasmic signalling sequence. Stimulation of chimeric receptors with plate bound CD33.

Figure 4: Blastogenesis induced by antigen specific stimulation of chimeric receptor proteins expressed in resting $CD8^+$ human primary T cells having cytoplasmic signalling domains comprising a cytoplasmic signalling sequence derived from CD134 or ICOS in addition to a TCRζ cytoplasmic signalling sequence. Stimulation of chimeric receptors with plate bound CD33.

Figure 5: Ki67 expression induced by antigen specific stimulation of chimeric receptor proteins expressed in resting $CD8^+$ human primary T cells having cytoplasmic signalling domains comprising a cytoplasmic signalling sequence derived from CD134 or ICOS in addition to a TCRζ cytoplasmic signalling sequence Stimulation of chimeric receptors with plate bound CD33.

Figure 6: Cytokine production elicited by antigen specific stimulation of chimeric receptor proteins expressed in resting total human primary T cells having cytoplasmic signalling domains comprising two secondary signalling sequences and one primary signalling sequence wherein one of the secondary signalling sequences is derived from CD134. Stimulation of chimeric receptors with plate bound CD33.

Figure 7: The effect of including costimulatory regions on the ability of chimeric receptors, expressed in resting T-cells, to mediate target cell killing.

EXAMPLES

Example 1: Construction of receptor cloning cassette

To facilitate construction and analysis of signalling region combinations in a chimeric receptor format, a cloning cassette was devised in pBluescript SK+ (Stratagene) and pcDNA3 (Invitrogen). This cloning cassette consists of a binding component cassette and a spacer/transmembrane cassette.

This new cassette system is shown in Figure 1. The binding component has 5' (relative to coding direction) Not I and Hind III restriction sites and a 3' (again relative to coding direction) Spe I restriction site. The extracellular spacer is flanked by a Spe I site (therefore encoding Thr, Ser at the 5' end) and a Nar I site (therefore encoding Gly, Ala at the 3' end). The transmembrane component is flanked by a Nar I site at its 5'end (therefore encoding Gly, Ala) and by Mlu I (therefore encoding Thr, Arg) and BamH I sites (therefore encoding Gly, Ser) at the 3' end. The cytoplasmic signalling domain may be cloned in-frame into the BamH I site. Following this BamH I site there is a stop codon for transcription termination and there is also an EcoR I site situated downstream of this to facilitate the subsequent rescue of whole constructs.

a) Binding Component Cassette (Hind III to Spe I) This consists of the V_L and V_H regions of an anti-CD33 antibody joined by a short linker sequence. The V_L region was PCR cloned from hP67scFv (WO97/23613) with oligos A5267 and F22785 and digested with restriction enzymes to generate a Hind III to Bgl II fragment. The V_H region was PCR cloned from hP67scFv (WO97/23613) with oligos F22786 and F22787 and digested with restriction enzymes to generate a Bgl II to SpeI fragment. These two fragments were then co-ligated together with the linker sequence into a cloning vector. The linker was formed by annealing oligos F22966 and F22967 which have 5' phosphate groups: these annealed oligos were compatible with Bgl II overhangs but did not reform the Bgl II site on ligation.

b) h.28 spacer/ CD28 transmembrane Cassette (Spe I to EcoRI with internal BamHI site for cloning signalling cassettes prior to a stop codon) The extracellular spacer component h.28, consists of residues 234 to 243 of human IgG1 hinge and residues 118 to 134 of human CD28. The transmembrane component consists of residues 135 to 161 of human CD28 (Aruffo & Seed 1987).

To generate this cassette, a 200bp fragment was PCR assembled using the methods described in Example 1 (b) of WO02/33101. This fragment starts with a SpeI site and consists of the extracellular spacer h.CD28, the human CD28 transmembrane region, a BamHI site, a stop codon and finishes with an EcoRI site.

c) G1 spacer/TCR ζ transmembrane Cassette (Spe I to EcoRI with internal BamHI site for cloning signalling cassettes prior to a stop codon)

The extracellular spacer termed "G1" consists of the hinge CH2 and CH3 regions, residues 134 to 478 of human IgG1 (Kabat *et al.*, 1991).

The G1 spacer was cloned together with the human TCR ζ transmembrane region, residues 10 to 30 (Weissman *et al.*, 1998; Moingeon *et al.*, 1990), with an internal NarI site between spacer and transmembrane region so that they may be exchanged individually. The hinge region, CH2 and CH3 were cloned from a previously reported construct, (Finney *et al.*, 1998) with 5' oligo F24675 and 3' oligo F24676. F24675 is complementary to eight amino acids of the hinge region and adds a 5' SpeI site. F24676 is complementary to ten amino acids of CH3 and

additionally adds the internal NarI site, the twenty one amino acids of the TCR ζ transmembrane region and a 3' MluI site. This PCR fragment was restricted with SpeI and MluI and substituted for the h.28 spacer and the CD28 transmembrane region in a scFv/h.28/CD28TM/- vector. Alternatively, this PCR fragment was restricted with Spe I and Nar I and substituted for the h.28 spacer only in a scFv/h.28/CD28TM/- vector.

Example 2: Construction of chimeric receptors with different combinations of cytoplasmic signalling sequences

Cytoplasmic signalling sequences were then cloned into the BamHI site of the above cassette. Because each signalling sequence is on a BclI to BamHI fragment, directional cloning of these sequences retains a BamHI site at the 3' end only, allowing subsequent cloning of a second and third cytoplasmic signalling sequence. This cloning method generates a 2 amino acid spacer (Gly,Ser) between each signalling sequence.

a) Zeta Signalling Cassette (Bcl I to BamH I fragment) This consists of residues 31 to 142 of human TCRζ chain (Weissman *et al* 1988, Moingeon *et al.*, 1990) and was PCR cloned using oligos F34729 and F34730 from pHMF492 (a zeta chimeric receptor described in International Patent application PCT/GB00/01456) and digested with restriction enzymes BclI and BamHI.

b) CD28 Signalling Cassette (Bcl I to BamH I fragment) This comprises the intracellular component of human CD28 of consists residues 162 to 202 of CD28 (Aruffo & Seed 1987). This component was formed by annealing oligos with 5' phosphate groups B0735 and B0736: the single stranded overhangs of these annealed oligos form a 5' BclI half site and a 3' BamHI half site.

c) CD134 Signalling Cassette (Bcl I to BamH I fragment) This consists of residues 213 to 249 comprising the intracellular component of human CD134 (Latza *et al.*, 1994) and was formed by annealing oligos with 5' phosphate groups F18605 and F18606: the single stranded overhangs of these annealed oligos form a 5' BclI half site and a 3' BamHI half site.

d) ICOS Signalling Cassette (Bcl I to BamH I fragment) This consists of residues 166 to 199 comprising the intracellular component of human ICOS (Hutloff *et al.*, 1999) and was formed by annealing oligos with 5' phosphate groups F34731 and F34732: the single stranded overhangs of these annealed oligos form a 5' BclI half site and a 3' BamHI half site.

The nomenclature used to describe the receptors of the present invention is the scFv followed by the spacer region and then the signalling region. When the signalling region contains more than one component, the membrane proximal one is listed first. For example, P67/h.28/Zeta represents a receptor with the P67 scFv binding region, the h.28 extracellular spacer and the TCRζ signalling region. P67/h.28/CD28-Zeta-CD134 represents a receptor with the P67 scFv binding region, the h.28 extracellular spacer and the CD28 signalling region fused to the TCRζ signalling region fused to the CD134 signalling region, with CD28 membrane proximal.

Example 3: Analysis of receptors

a) Construction of expression plasmids. The chimeric receptor constructs were sub-cloned from pBluescript KS+ into the expression vector pQBI-CMV5 (CT) (QBIOgene) on a Hind III to EcoR I restriction fragment. The empty expression vector (i.e the base vector lacking in chimeric receptor genes) is used as a negative control.

b)Purification of human Peripheral blood mononuclear cells (PBMC) and isolation of human primary T cell subsets. Whole blood was taken from healthy volunteers by venous puncture using heparinised vacutainers (BD Biosciences,UK), diluted 1/3 in Phosphate buffered saline (PBS)/0.5% Foetal calf serum (FCS) and carefully layered over 15ml of Ficoll-paque™PLUS (Amersham Biosciences, Sweden) in a 50ml tube and centrifuged at 450g for 30 minutes, letting the rotor stop without brake. Cells at the interface of the gradient were carefully removed and diluted greater than five-fold with PBS and centrifuged again at 300g for ten minutes. Cells were then given a final PBS wash, centrifuging at 200g for ten minutes. Cells were counted and resuspended in the appropriate volume of MACs buffer (PBS/0.5% BSA/2mM EDTA) for magnetic bead depletion. The desired population of cells were then purified from these PBMC by depletion on LS columns using a VarioMacs™

magnetic seperator and either a total T cell isolation kit, a $CD4^+$ T cell or a $CD8^+$ T cell isolation kit exactly as directed by the manufacturer's (MiltenyiBiotec, GmbH).

c)Transfection of human primary T cells using the Nucleofector™device

4-8 x 10^6 purified human primary T cells were resuspended in 100µl of the manufacturer's supplied "T cell solution", mixed with 5-10µg plasmid DNA in a supplied cuvette and subjected to programme U-13 in the Nucleofector™ electroporation device (amaxa biosystems, GmbH). Cells were immediately removed from the cuvette and cultured in RPMI supplemented with 10% FCS, 4mM glutamine and 1% penicillin/streptomycin (GIBCO™, U.K.) at 37°C/8% CO_2.

d) Stimulation of transfected human primary T cells

Anti-human CD3ε antibody, OKT3 (ATCC), was purified from hybridoma supernatant on a protein A-Sepharose column (Pharmacia, NJ), eluting protein with a pH gradient of buffers from pH 9.0 (0.15M Na_2HPO_4) to pH 2.0 (0.1M citric acid). The eluted antibody was neutralised with 2M Tris/HCl, pH 8.5 and then concentrated and buffer-exchanged into PBS, pH 7.0, using a Diafilter rig (Amicon, UK). Soluble human CD33 was purified from supernatant from an NS0 stable cell line (Finney *et al.*, 1998) by affinity chromatography on a mouse P67 affinity column (Celltech). The protein was eluted with 0.1M citric acid buffer and then neutralised with 2M Tris/HCl, pH 8.5. Soluble CD33 was concentrated and buffer-exchanged into PBS, pH 7.0, using a Diafilter rig (Amicon, UK). 2 to 5 x 10^6 T cells per well of a 96 well plate were stimulated plates coated with either OKT3 or CD33 or both. Plates were coated with 2µg/ml OKT3 antibody or / and CD33 in carbonate buffer (0.1M $NaHCO_3$, pH 9.6) for at least 2 hours at room temperature. These plates were then washed with PBS and dried prior to addition of cells.
Cells were stimulated for 48 hours for the analysis of cytokine production, for 5 days for the analysis of changes in cell morphology and for 6 days for analysis of Ki67 expression.

e)Cytokine ELISAs Assays for human IL-2, IFN-γ, TNF-α, and GM-CSF were performed using Duoset kits as described by the manufacturer (R&D systems, UK). Nunc™ Maxisorp microtitre plates were incubated with 4µg/ml of the capture

antibody diluted in PBS overnight at room temperature. These plates were then washed four times with PBS/0.1% Tween and blocked for two hours with PBS/1%BSA/5% sucrose. 100 µl of sample supernatant and positive control protein standard titrations were then incubated for two hours at room temperature, before washing the plates four times as before. The recommended dilution of biotinylated detection antibody in PBS was incubated for one hour, plates washed again and then incubated with Streptavidin-HRP (Horse radish peroxidase) for 15 minutes. Plates were washed again and colour detected with tetramethylbenzidine (TMB) substrate followed by absorbance reading at 630nm using a Labsystems Multiskan Ex plate reader (Labsytems, UK). Standard curves were constructed and data analysed using Genesis II software (Labsytems, UK).

f) Analysis of changes in cell morphology and proliferation

Changes in cell morphology were measured by washing cells in FACS buffer (PBS/ 5% FCS), and forward scatter versus side scatter dot plots analysed using a FACScalibur flow cytometer (BD Biosciences). Data was acquired and analysed with CellQuest software (BD Biosciences).

Proliferation was analysed by examining the expression level of the proliferation marker, Ki67. 1 to 2 x 10^5 cells were washed with 10 to 20 volumes of FACS buffer (PBS/ 5% FCS) in 5ml tubes, and then resuspended in 100µl cytofix-cytoperm™ (BD Biosciences), and incubated at 4°C for 20 minutes. The cells were washed with Perm-wash™ (BD Biosciences) before resuspension in 200µl of the same buffer. 10 µl of fluorscein-labelled anti Ki67 antibody (Dako) antibody was added, and cells incubated for 30 minutes at 4°C. The cells were then washed and resuspended in FACS buffer (PBS/ 5% FCS), and analysed using a FACScalibur flow cytometer (BD Biosciences). Data was acquired and analysed with CellQuest software (BD Biosciences).

g) Target Cell Lysis

T cells were isolated and transfected as described above, left to rest for 3 h after transfection and then 2X10^5 cells stimulated by incubation in round-bottom 96 well plates with target cells at an effector:target cell ratio of 5:1, 10:1, 20:1, 40:1 and 80:1. Target cells were mouse myeloma NS0 cells transfected with a human CD33

construct (antigen positive) or control vector (antigen negative). These target cells were labelled by incubation with 5μM CFSE (Molecular Probes, Eugene, OR) at 37°C for 15 min in RPMI media and then washed twice with PBS. Effector T cells and target cells were incubated for 40 h in a humidified 37°C incubator, and then target cell viability was analysed by flow cytometry. CFSE-labelled target cells were distinguished from effector cells by their FL1 chanel fluorescence, and viable cells were detected by their ability to exclude propidiun iodide, detected on the FL3 channel .The percentage of target cell lysis was calculated to be (percentage viable target cells in the absence of effector cells) – (percentage viable target cells in the presence of effector cells).

Example 4: Results

Figure 2 shows that when expressed in resting human primary CD4+ T cells, chimeric receptors comprising a CD134 or ICOS signalling region, can co-stimulate OKT3-mediated IL-2 production on binding of ligand (CD33) binding to the extracellular ligand binding domain (P67 scFv). These receptors are compared to a matched control chimeric receptor with no signalling region.

Figure 3 shows that when a cytoplasmic signalling sequence from CD134 or ICOS is incorporated in a cytoplasmic signalling region with a TCR zeta primary signalling region of a chimeric receptor, activation of this chimeric receptor, by ligand binding to the extracellular ligand binding domain, results in cellular activation, as indicated by the production of IL-2 TNF-α IFN-γ and GM-CSF. This cellular activation is greatly enhanced compared to to a matched control chimeric receptor with only a TCR zeta primary signalling region.

Figure 4 shows that when a cytoplasmic signalling sequence from CD134 or ICOS is incorporated in a cytoplasmic signalling region with a TCR zeta primary signalling region of a chimeric receptor, activation of this chimeric receptor, by ligand binding to the extracellular ligand binding domain, results in cellular activation, as indicated by changes in cell morphology. Cells expressing chimeric receptors including either CD134 or ICOS in the signalling region, form T cell blasts on activation. These blasts are larger, diving cells. This cellular

activation is not seen with a matched control chimeric receptor with only a TCR zeta primary signalling region.

Figure 5 shows that when a cytoplasmic signalling sequence from CD134 or ICOS is incorporated in a cytoplasmic signalling region with a TCR zeta primary signalling region of a chimeric receptor, activation of this chimeric receptor, by ligand binding to the extracellular ligand binding domain, results in cellular activation, as indicated by expression of the proliferation marker, Ki67. Cells expressing chimeric receptors including either CD134 or ICOS in the signalling region proliferate on activation. This cellular activation is not seen with a matched control chimeric receptor with only a TCR zeta primary signalling region.

Figures 2-5 illustrate the surprising finding that CD134 and ICOS are capable of activating resting T cells in the absence of CD28 signalling.

Figure 6 shows that when a cytoplasmic signalling sequence from CD134 is incorporated in a cytoplasmic signalling region with a TCR zeta primary signalling region and a CD28 secondary signalling region of a chimeric receptor, activation of this chimeric receptor, by ligand binding to the extracellular ligand binding domain, results in enhanced cellular activation, as indicated by the production of IL-2, GMCSF and IFN-γ. This cellular activation is enhanced compared to a matched control chimeric receptor with only a TCR zeta primary signalling region and to a control chimeric receptor with a TCR zeta primary signalling region and a CD28 secondary signalling region.

Figure 7 shows that the effect of including costimulatory regions on the ability of chimeric receptors, expressed in resting T cells, to mediate target cell killing. A chimeric receptor comprising zeta signalling alone was compared to receptors including CD28, CD134 or ICOS in series with zeta. No target cell killing was observed with control (vector) transfected T cells or with antigen negative cells. The zeta receptor mediated antigen specific target cell lysis within 40 hrs of stimulation at effector to target cell ratios of greater than 10:1. This target cell lysis was enhanced by the inclusion of both costimulatory regions normally expressed in

resting T cells (CD28) and those not normally expressed until after TCR and CD28 stimulation (CD134 and ICOS). Surprisingly ICOS enhanced target cell killing to a far greater degree than CD28.

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